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Although many mechanisms have been proposed to account for the anti-tumor activities of therapeutic antibodies, including extended half-life, blockade of signaling pathways, activation of apoptosis and effector-cell-mediated cytotoxicity, we have demonstrated (**Clynes RA,** Towers TL, Presta LG, Ravetch JV, Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets. *Nature Medicine* 6:443-446 (2000)) that engagement of Fcgamma receptors on effector cells is a dominant component of the *in vivo* activity of antibodies against tumors. Engagement of activating Fc receptors (FcRI and/or III) was required for the *in vivo* activity of mouse monoclonal antibodies and vaccines in syngenic melanoma models, as well as of humanized, clinically effective therapeutic mAbs Herceptin and Rituxan in breast cancer and lymphoma xenograft system). Mice deficient in the inhibitory receptor FcgammaRIIB showed much more antibody-dependent cell-mediated cytotoxicity; while in contrast, mice deficient in activating Fc receptors as well as antibodies engineered to disrupt Fc binding to those receptors were unable to arrest tumor growth *in vivo*.

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INTRODUCTION

We have completed year two of this funded project on track, having demonstrated in year 1 that monoclonal antitumor antibodies require FcR engagement for full activity in vivo. These findings were published in Nature Medicine. In year two we have generated novel transgenic mice that express Fc receptors in a lineage specific manner. These mice express activating Fc receptors in only myeloid cells or only NK cells. These mice will be invaluable to the investigation of the singular importance of individual Fc receptor- bearing cellular subsets to ADCC in vivo. We continue our mouse breeding and transgenic mouse production to generate genotypically unique mice that can be tested for involvement of specific FcR bearing cell types and specific apoptotic pathways in ADCC in vivo.

Statement of Work: Progress Report Year 1
Cytotoxic Mechanism of Tumor-Specific Antibodies:

1. **Characterization of the role of the cellular receptors for IgG (FcγRI, II and III) and complement in mediating tumor responses induced by anti-melanoma, lymphoma and breast carcinoma monoclonal antibodies.**

a) Analysis of anti-tumor responses in γ -/-, FcγRII -/-, FcγRIII -/- and C3 -/- athymic nude mice using Herceptin, Rituxan and anti-gp75 mAbs in breast cancer, lymphoma and melanoma models (months 1 to 6, 200 mice).

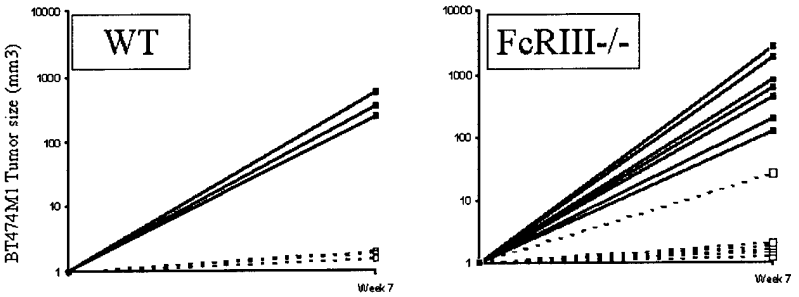
Progress Year 1: In work published in *Nature Medicine* (please see attached manuscript for details) we have determined that the activating Fc receptors (I and III) are required for the in vivo activity of antitumor antibodies. In addition the inhibitory receptor, FcRII, was found to modulate the potency of these antibodies, including the anti-breast cancer antibody, Herceptin. At 10% of the dose that was effective in wild-type mice, FcRII -/- mice were completely protected from tumor growth. The implications from these studies are profound and have provided the catalyst for major efforts in industry to generate anti-tumor antibodies which p[referentially recruit activating Fc receptors at the expense of the inhibitory Fc receptors.

Progress Year 2: We have bred the FcRIII -/- mice with athymic nu/nu mice to determine which activating Fc receptor (Type I or III) is required for ADCC in vivo. Our results below (Figure 1) shows that FcRIII is not required for Herceptin-mediated antitumor efficacy using BT474M1 xenografts. These mice still retain functional expression of the remaining activating Fc receptor, FcRI. Given our previous published findings that anti-tumor antibody protection is absent in FcR γ-/- mice which lack both activating Fc receptors FcRI and FcRIII, this new result with FcRIII-/- mice suggests that FcRIII is not necessary and FcRI is sufficient for ADCC in vivo. It however does not dismiss the possibility of redundant pathways in which either FcRI or III is sufficient. Further this provocative results suggests that NK-mediated ADCC is also not

necessary for antitumor antibody immunity since FcRIIII is the sole FcR expressed on this cell type.

In addition we are breeding the C3 -/- mice to the athymic nu/nu background to confirm our suspicions that complement plays little or no role in mediating cellular cytotoxicity in vivo.

FcRIII is Not Required for Anti-HER2 Mab Tumor Immunity



2. Identification of FcγR-bearing effector cells responsible for ADCC in vivo.

a)Reconstitution of ADCC phenotype by cell transfer of γ -/- with wild-type bone marrow, macrophages and NK cells (months 1 to 6, 120 mice).

b)Reconstitution of ADCC of γ -/- by tissue specific transgenes.

- Engineering of NK cell and Macrophage specific γ expressing plasmids (months 1 to 3)
- Demonstration of tissue specific reconstitution in transient and stable γ -/- transfectants (months 3 to 5).
- Generation of transgenic mice bearing tissue-specifc transgenes (months 5-12).
- Confirmation of tissue specific expression in transgenic mice (months 12-14)
- Breeding and analysis of TA99 anti-tumor antibody responses in transgenic mice (months 12-18, 40 mice)
- Generation of γ –/– nu/nu mice bearing γ transgenes by two rounds of mating with founder lines (months 12- 18)
- Breeding and analysis of Harceptin and Rituxan anti-tumor responses in γ –/– nu/nu mice bearing γ transgenes (months 18 to 36, 80 mice)
- Generation of transgenic mice bearing γ/humanFcγRIIIA transgenes and analysis in xenograft nu/nu models (months 24 to 48, 80 mice)

We have concentrated on genetic reconstitution as a first priority. We have successfully generated expression constructs which target lineage specific expression to NK cells and myeloid cells. These constructs have been injected into embryos and transgenic founder lines generated. Transgenic mice have been generated which harbor the FcRγ gene driven by either a granzyme promoter (NK cell specific) or the CD11b promoter (myeloid cell specific).

Identification of Cell Type and Receptor Class Responsible for ADCC in Vivo

Lineage Specific Reconstitution of FcR γ -/- Mice

Transgenic Construct	Lineage	FcR Expression
CD11b promoter | γ chain	Monocytes	mFcRI,III
Granzyme promoter | γ chain	NK cells	mFcRIII

Three founder Tg+ lines of each construct have been screened for lineage-specific expression. One of three lines of each transgenic construct exhibited no evidence of functional expression of FcRIII by flow cytometry and functional assays while the other two lines were positive expressors and were further screened. Functional analysis of the higher expressor of the remaining two lines is shown in Figure 2 for granzyme-γ and Figure 3 for CD11b-γ.

Granzyme promoter	γ chain

NK Cell Mediated ADCC is Reconstituted in Granzyme-γ Transgenic

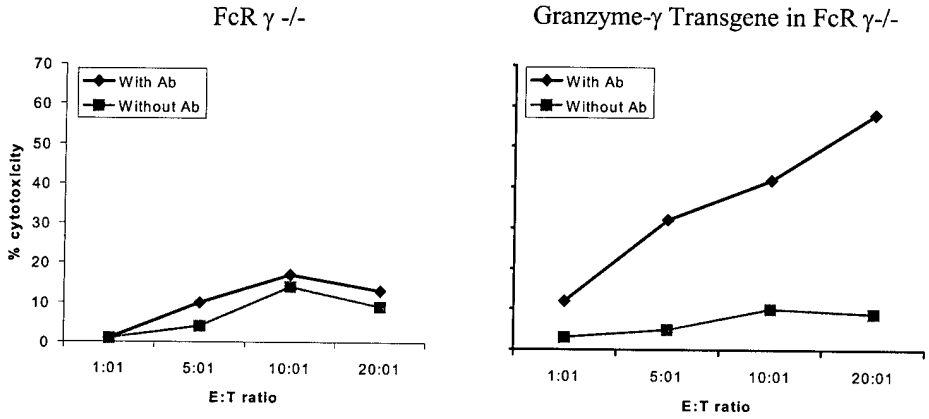


Figure 2: NK cells cultured from 10 day cultures of IL-2 stimulated nylon-wool non-adherent splenocytes were incubated with Chromium labeled TNP-derivitized EL-4 cells in the presence or absence of anti-TNP IgG2b antibodies for 4 hrs and % cytotoxicity determined.

Granzyme-γ NK cells were found to express FcRIII on 15% of IL-2 stimulated NK cells by flow cytometry, whereas FcRγ-/- NK cells were devoid of FcRIII expression and WT NK cells exhibited 90% expression (data not shown). However, despite this incomplete reconstitution of FcR expression in the NK population these cells were as good or better at mediating ADCC *in vitro* as WT NK cells (Figure 2 and data not shown). Lineage restricted expression in granzyme-γ transgenic mice has been confirmed by analysis of monocyte, neutrophil and B lymphocyte populations which do not express γ-chain by Western analysis (data not shown). Thus granzyme-γ transgenic mice are expected to provide an invaluable reagent to explore the consequences of NK-mediated ADCC *in vivo*, in particular antibody-mediated tumor immunity. They are successfully breeding and will be challenged with B16 melanoma +/- the anti-melanoma antibody TA99. The ability of TA99 to prevent lung metatstases in this model will define the unique contribution of NK cell FcR-mediated ADCC *in vivo*.

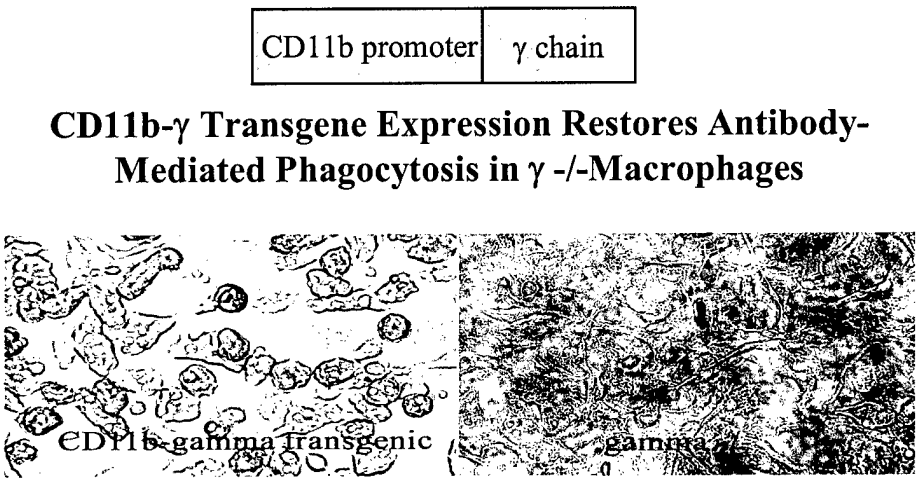


Figure 3: Thioglycollat-elicited macrophages were incubated with IgG-coated SRBCs for 30 minutes and uningested RBCs lysed by osmotic shock. CD11b-γ macrophages (left) but not FcR γ -/- (right) macrophages exhibited WT levels of phagocytosis.

Analysis of CD11b-γ transgenic mice revealed that FcR-mediated phagocytosis had been reconstituted in FcRγ-/- macrophages but not in granzyme-γ tg animals. In contrast NK cells from CD11b-γ transgenic mice did not express FcRIII and were unable to perform ADCC demonstrating that this myeloid-specific promoter was not being expressed in NK cells (data not shown). Western analysis using rabbit-anti γ IgG revealed WT levels of gamma expression in CD11b-γ macrophages but no detectable expression in lymphoid cells. Thus these mice will be useful to address the specific contribution of FcR-mediated macrophage activation to antitumor antibody efficacy *in vivo*. These mice are breeding well and will be tested for the contribution of macrophages in antitumor antibody mediated prevention of B16 melanoma lung metastases.

We are currently breeding these animals (four founder lines each) and will assay the mice for lineage specific expression of then transgenes when the mice are available in sufficient numbers. Our initial studies will use the melanoma model as the transgenic mice were generated in the C57Bl.6 background which is syngenic with murine melanoma cell lines. Once lineage specific expression is confirmed the mice will be read onto the nude background and onto the HER2-transgnic background for breast cancer models.

3. Dependence of antibody-mediated cytotoxicity on Fas-mediated target cell apoptosis.

- Analysis of Herceptin and Rituxan anti-tumor responses in athymic nude gld/gld and perforin-deficient mice (months 1 to 12, 80 mice)
- Construction of fas and anti-fas expression constructs (months 1 to 3)
- Selection of fas and anti-fas stable transfectants using B16F10, Daudi and BT474M1 cell lines (months 4 to 8)
- Tumor susceptibility studies of fas and anti-fas stable transfectants with Herceptin, Rituxan and TA99 antitumor antibodies (months 8 to 20, 120 mice)
- Analysis of fas up-regulation BT474M1 breast carcinoma by cytotoxic agents and radiotherapy (months 1 to 6)
- Established tumor response studies with combined therapy: Herceptin and cytotoxic agents (months 6 to 24, 100 mice).

We have had breeding problems with groups of gld/gld nu/nu mice to test the role of Fas in mediating the anti-tumor effects of Herceptin.

We have failed to generate stable cell lines expressing fas and anti-fas genes in all lines tested implying these constructs were toxic. Therefore we have approached this issue through alternative methods and have generated stable transfectants with the anti-apoptotic gene c-FLIP. We are currently screening clones for expression.
In the interim since this grant was written it has been established that chemotherapeutic agent and antitumor antibodies can act synergistically in vivo although the mechanisms are still unclear.

Research Accomplishments:

• We established a general requirement for FcR activation for the in vivo activity of antitumor antibodies including the clinical therapeutic mAbs, Herceptin and Rituxan.
• We established that the inhibitory receptor FcRII dramatically reduces the in vivo acitivity of antitumor antibodies including the Herceptin and Rituxan.
• We have determined that FcRIII engagement is not required for the anti-tumor activity of Herceptin suggesting that NK cells are not involved and that FcRI expressed on myeloid cells may be sufficient to induce fully effective ADCC in vivo.
• We have generated transgenic mice that express activating Fc receptors ONLY in NK cells or myeloid cells. Tissue specific expression and functional reconstitution has been confirmed; NK cell FcR-mediated ADCC in NK-γ tg+ and FcR-mediated macrophage

phagocytosis in Mac-γ tg+ mice. These mice will be valuable tools to determine the role of these individual cell types in ADCC in vivo.

Reportable Outcomes: Year 1/Year 2

Publications:

Year 1Clynes RA, Towers TL, Presta LG, Ravetch JV, Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets. *Nature Medicine* 6:443-446 (2000).

Grants/Awards Received

Year 1 Cancer Research Institute Investigator Award 2000
Year 2 Charles Carrington Award in Biomedical Research 2000
Year 2 Kimmel Cancer Investigator Award 2001
Year 2 Speaker Biomedical Research Award 2001
Year 2 R01 NCI budget pending

Invited Meetings

Year 1 Invited Speaker Keystone Symposia 2000 (Cellular Immunity and Immunotherapy of Cancer)
Year 2 Stanford University Department of Pathology Invited Lecturer (Charles Carrington Award Recipient)

Conclusions

We are pleased that our work has been recognized in Nature Medicine (please see commentary in attached appendix) for its significance in providing a unifying general mechanism for antitumor antibodies. We are excited to pursue this mechanism in further detail beginning with the identification of the required cellular effectors responsible for Fc receptor mediated cellular cytotoxicity in vivo.